REMARKS

Claims 1, 2, 30, and 31 have been amended to replace the term "standard conditions" with the phrase "stringent hybridization and stringent washing conditions." Support for this amendment is found in the specification at, for example, page 16, lines 1-15.

It is submitted that no new matter has been introduced by the foregoing amendments.

35 U.S.C. § 112, Second Paragraph, Rejections:

Claims 1-3, 20-22, 25-26, and 29-31 have been rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. (Paper No. 20060705 at 2).

In making the rejection, the Examiner asserted that the phrase "hybridizes under standard conditions" renders the claims indefinite. (*Id.*). The Examiner further asserted that "[t]he claims do not explicitly state the conditions which Applicants call 'standard.'" (*Id.* at 2-3). The Examiner also asserted that "Applicants refer to Sambrook in an exemplary way and do not quote any page presenting the conditions. It is unknown what conditions considered "standard" in the art are included and/or excluded from the scope of the claims." (*Id.* at 3).

Initially, we note that claim 26 has been previously cancelled; therefore, the rejection is moot as to claim 26.

With a view towards furthering prosecution, claim 1 (from which claims 2-3, 20-22, 25, 28, and 29 depend), claim 30, and claim 31 have been amended to replace the term "standard conditions" with the phrase "stringent hybridization and stringent washing conditions." The specification as filed provides details as to how this

phrase is to be interpreted, *see, e.g.*, page 16, lines 9-15. For exact details of these hybridization procedures, the specification cites to Sambrook *et al.*, Molecular Cloning (2nd ed.), Cold Spring Harbor Laboratory Press 1989, New York. For example, Sambrook provides on page 9.52 a complete protocol for stringent hybridization and stringent washing conditions. A copy of this protocol is attached as Exhibit 1.

In view of the amendments and the disclosure in the specification to look to Sambrook for the particulars of the high stringency wash and hybridization, it is respectfully submitted that one skilled in this art would readily understand the scope of the claims.

For the reasons set forth above, it is believed that the rejection of claims 1-3, 20-22, 25, and 29-31 is rendered moot. Accordingly, withdrawal of the rejection is respectfully requested.

35 U.S.C. § 112, First Paragraph, Rejections:

Claims 1-3, 20-22, 25, 28, and 30-31 have been rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. (Paper No. 20060705 at 3).

In making the rejection, the Examiner asserted that "the disclosure does not teach the hybridization conditions to be used in selection of DNA molecules of [the] invention, [and therefore] claims 1-3, 20-22, 25, 28, 30-31 as amended are not enabled and thus rejected." (*Id.* (original emphasis)).

In response to Applicants' remarks submitted April 20, 2006, the Examiner stated: "Applicants[‘] emphasize that the degree of homology between the enzymes disclosed and identified as SEQ ID NO: 5, 6, 7, and 8 is at least 80%, and any known alcohol dehydrogenase activity was in the range of 26 to 31%." (*Id.* at 3). The

Examiner then stated that: "Applicants' argument has been fully considered but is found not persuasive, because it is an argument pertaining to the question of existence of prior art, and not the question of enablement." (*Id.* at 4).

Initially, we note that it is the Examiner's burden to demonstrate that a specification is not sufficiently enabling. *In re Marzocchi*, 169 USPQ 367, 369 (CCPA 1971). To carry her burden, the Examiner must identify and clearly articulate the factual bases and supporting evidence that allegedly establish that undue experimentation would be required to carry out the claimed invention. *Id.* at 370.

With a view towards furthering prosecution, claim 1 (from which claims 2-3, 20-22, 25, 28, and 29 depend), claim 30, and claim 31 have been amended to replace the term "standard conditions" with the phrase "stringent hybridization and stringent washing conditions."

We note that the phrase "hybridizes under stringent hybridization and stringent washing conditions" is an art-recognized phrase that one skilled in the art would understand, even without further guidance from the specification. However, as discussed above, the specification as filed (at page 16, lines 9-15) discloses how this term is to be interpreted by reference to the art recognized Sambrook book. It is respectfully submitted that one skilled in the art armed with the presently amended claims, the specification, and Sambrook would have very quickly found on page 9.52 of Sambrook a complete protocol for stringent hybridization and stringent washing conditions as disclosed and claimed. For the preparation of the hybridization solution, Sambrook also discusses on page 9.47, item 1, that aqueous solutions and solutions of 50% formamide are both solvents that show excellent results. Therefore, the Examiner's statement that "the disclosure does not teach the hybridization conditions to

be used in selection of DNA molecules of [the] invention" is ***misplaced***. (Paper No. 20060705 at 3). For this reason alone, the rejection should be withdrawn.

Moreover, the specification discloses that the nearest homologues of Enzyme B (SEQ ID NO: 8) exhibit a maximum homology of 26-31% with known enzymes (page 34, line 20 to page 35, line 4):

> Homology search of Enzymes A, A', A" and B revealed that Enzymes A, A', A" and B showed rather low homology (26-31% homology through the polypeptides) with several quino-proteins including alcohol dehydrogenase of *Acetobacter aceti* (T. Inoue et al., J. Bacteriol. 171: 3115-3122) or *Acetobacter polyoxogenes* (T. Tamaki et al., B.B.A., 1088: 292-300), and methanol dehydrogenase of *Paracoccus denitrificans* (N. Harms et al., J. Bacteriol., 169: 3966-3975), *Methylobacterium organophilum* (S.M. Machlin et al., J. Bacteriol., 170: 4739-4747), or *Methylobacterium extorquens* (D.J. Anderson et al., Gene 90: 171-176).

One skilled in the art would immediately recognize that the DNA encoding such known enzymes would ***not*** hybridize under "***stringent hybridization and stringent washing conditions***" to the DNA encoding SEQ ID NO: 8 (*i.e.*, a DNA according to SEQ ID NO: 4). Thus, such known enzymes would not fall within the scope of the currently claimed subject matter.

Table 7 of the specification details the degree of homology between the AADH (Alcohol/Aldehyde Dehydrogenases) of SEQ ID NO: 8 and three other amino acid sequences having AADH activity, *i.e.*, SEQ ID NOS: 5, 6 and 7, which are disclosed throughout the specification. The results in Table 7 demonstrate that a homology of at least 80% was detected:

Table 7. Homologies of amino acid sequences among AADHs.

	Enzyme A	Enzyme A'	Enzyme A"	Enzyme B
Enzyme A	100	_	_	_
Enzyme A'	89	100	_	_
Enzyme A"	85	86	100	_
Enzyme B	83	82	81	100

(See specification at page 34, lines 15-20). As discussed above, the next highest homology between Enzymes A, A', A", and B to enzymes with known alcohol or methanol dehydrogenase activity was in the range of 26 to 31%. (See Specification at page 34, line 20 to page 35, line 4). Thus, the data in Table 7 clearly provides evidence that the Applicants enabled the full scope of the amended claims by unambiguously identifying enzymes having highly homologous polypeptide sequences and sharing a common function -- AADH activity.

The Examiner's statement that "Applicants' argument has been fully considered but is found not persuasive, because it is an argument pertaining to the question of existence of prior art, and not the question of enablement" is not understood. (Paper No. 20060705 at 4). The previous argument made no reference to any prior art. Rather, the argument pertained to and still pertains to the degree of experimentation needed to identify enzymes having highly homologous polypeptide sequences and sharing AADH activity.

As is well accepted, even a "considerable amount" of experimentation is permissible if it is merely routine or if the specification provides a reasonable amount of guidance. MPEP § 2164.05 and *In re Wands*, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). Here, the specification provides ample guidance, both by disclosing the degree of

homology between the AADH of SEQ ID NO: 8 and three other amino acid sequences having AADH activity, *i.e.*, SEQ ID NOS: 5, 6 and 7 and by requiring "stringent hybridization" and "stringent washing conditions." Accordingly, it is respectfully submitted that undue experimentation would not be required to carry out the currently claimed invention. For this additional reason, this rejection should be withdrawn.

Claims 2-3 have also been rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. (Paper No. 20060705 at 4).

In making the rejection, the Examiner asserted that claims 2 and 3 "do[ ] not reasonably provide enablement for an enzyme that comprises a combination of at least two amino acids sequences each of said sequences being selected from the group of SEQ ID NO: 8 and SEQ ID NO: 5 and amino acid sequences encoded by DNA sequences hybridizing under standard conditions with DNA molecules according to SEQ ID NO:4 or 1." (*Id.* (original emphasis)). The Examiner, however, acknowledged that claims 2 and 3 are "enabling for the plasmid comprising genes encoding SEQ ID NO: 5 and SEQ ID NO: 8 (plasmids pSSAB201 and pSSBA201)." (*Id.*).

In response to Applicants' remarks submitted April 20, 2006, the Examiner stated "without further guidance on the part of Applicants related to the structure of chimeric enzymes, one skilled in the art is forced to construct numerous combination[s] of disclosed sequences and/or sequences that are hybridizing to SEQ ID NO:1 or SEQ ID NO:4 under indefinite conditions, ..." (*Id.* at 5 (original emphasis)). The Examiner further stated that "[t]he figures are schematic and for that reason do not identify the details of structure, i.e., sequences, of claimed hybrid molecules." (*Id.*).

With a view towards furthering prosecution, claims 2 and 3 have been amended. Claim 1 (from which claims 2 and 3 depend) has been amended to recite

"stringent hybridization and stringent washing conditions" instead of "standard conditions." Therefore, the Examiner's concern regarding "indefinite conditions" has been rendered moot. (*Id.*)

In view of this amendment, we note that the construction of the currently claimed chimeric nucleic acid molecules and polypeptides is enabled and specifically disclosed in the specification at, for example, Examples 14 and 15 and in Figures 2, 3, 4, 7, and 8. The Examiner's statement that "figures [2, 3, 4, 7, and 8] are schematic and for that reason do not identify the details of structure, i.e., sequences, of claimed hybrid molecules" misses the point. (*Id.*). These figures must considered, not in isolation, but in conjunction with the specification as a whole and what one skilled in the art would learn from them. For example, the specification discloses the enzymatic activity of these constructs (*see, e.g.*, Figure 11). Furthermore, the specification also discloses in Tables 11 and 12 comparisons of the substrate specificities of the claimed enzymes. In view of the amended claims, the extensive disclosure in the specification, as well as the above-identified Tables and drawings, one skilled in this art would have been able to make and use what is claimed. Thus, the skilled person is ***not*** left "without a[ny] further guidance" as asserted by the Examiner. (Paper No. 20060705 at 5). In sum, the specification clearly enables the full scope of the currently claimed chimeric enzymes (*i.e.*, the chimeric enzymes identified as Enzyme B (*i.e.*, SEQ ID NO: 8) and Enzyme A (*i.e.*, SEQ ID NO: 5) and the chimeric enzymes encoded by DNA sequences hybridizing under "stringent hybridization and stringent washing conditions" with DNA molecules according to SEQ ID: 4 or 1).

In view of the foregoing, it is respectfully submitted that the rejection has been rendered moot. Accordingly, withdrawal of the rejection is respectfully requested.

For the foregoing reasons, favorable action on the merits, including entry of the amendments, withdrawal of the rejections, and allowance of all the claims, respectfully are requested. If the Examiner has any questions regarding this paper, please contact the undersigned attorney.

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop AF, Commissioner for Patents, P.O. Box. 1450 Alexandria, VA 22313-1450, on January 12, 2007.

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Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

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Cold Spring Harbor Laboratory Press
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HYBRIDIZATION OF RADIOLABELED PROBES TO IMMOBILIZED NUCLEIC ACIDS

There are many methods available to hybridize radioactive probes in solution to nucleic acids immobilized on solid supports such as nitrocellulose filters or nylon membranes. These methods differ in the following respects:

- Solvent and temperature used (e.g., 68°C in aqueous solution or 42°C in 50% formamide)
- Volume of solvent and length of hybridization (large volumes for periods as long as 3 days or minimal volumes for periods as short as 4 hours)
- Degree and method of agitation (continuous shaking or stationary)
- Use of agents such as Denhardt's reagent or BLOTTO to block the non-specific attachment of the probe to the surface of the solid matrix
- Concentration of the labeled probe and its specific activity
- Use of compounds, such as dextran sulfate (Wahl et al. 1979) or polyethylene glycol (Renz and Kurz 1984; Amasino 1986), that increase the rate of reassociation of nucleic acids
- Stringency of washing following the hybridization

Although the choice depends to a large extent on personal preference, we offer the following guidelines for choosing among the various methods available.

1. Hybridization reactions in 50% formamide at 42°C are less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. However, it has been found that the kinetics of hybridization in 80% formamide are approximately four times slower than in aqueous solution (Casey and Davidson 1977). Assuming a linear relationship between the rate of hybridization and the formamide concentration, the rate in 50% formamide should be two to three times slower than the rate in aqueous solution. Both types of solvents give excellent results and neither has a clear-cut advantage over the other.
2. The smaller the volume of hybridization solution, the better. In small volumes of solution, the kinetics of nucleic acid reassociation are faster and the amount of probe needed can be reduced so that the DNA on the filter acts as the driver for the reaction. However, it is essential that sufficient liquid be present for the filters to remain covered at all times by a film of the hybridization solution.
3. Continual movement of the probe solution across the filter is unnecessary, even for a reaction driven by the DNA immobilized on the filter. However, if a large number of filters are hybridized simultaneously, agitation is advisable to prevent the filters from adhering to one another.
4. The kinetics of the hybridization reaction are difficult to predict from theoretical considerations, partly because the exact concentration of the

immobilized nucleic acid and its availability for hybridization are unknown. When using probes that have the capacity to self-anneal (e.g., nick-translated double-stranded DNA), the following rule of thumb is useful: Allow the hybridization to proceed for a time sufficient to enable the probe in solution to achieve $1–3 \times C_o t_{1/2}$. In 10 ml of hybridization solution, 1 μg of a probe of 5-kb complexity will reach $C_o t_{1/2}$ in 2 hours. To determine the time of half-renaturation for any other probe, simply enter the appropriate values into the following equation:

$$1/x \times y/5 \times z/10 \times 2 = \text{number of hours to achieve } C_o t_{1/2}$$

where x = the weight of the probe added (in micrograms), y = its complexity (for most probes, complexity is proportional to the length of the probe in kilobases), and z = the volume of the reaction (in milliliters).

After hybridization to $3 \times C_o t_{1/2}$ has been reached, the amount of probe available for additional hybridization to the filter is negligible. For probes that do not have the capacity to self-anneal (e.g., RNA probes synthesized in vitro by bacteriophage-encoded DNA-dependent RNA polymerases; see Chapter 10), the hybridization time may be shortened, since the lack of a competing reaction in the solution favors hybridization of the probe to the DNA immobilized on the filter.

5. Several different types of agents can be used to block the nonspecific attachment of the probe to the surface of the filter. These include Denhardt's reagent (Denhardt 1966), heparin (Singh and Jones 1984), and nonfat dried milk (Johnson et al. 1984). Frequently, these agents are used in combination with denatured, fragmented salmon sperm or yeast DNA and detergents such as SDS. In our experience, virtually complete suppression of background hybridization is obtained by prehybridizing filters with a blocking agent consisting of 5× Denhardt's reagent, 0.5% SDS, and 100 μg/ml denatured, fragmented DNA. We recommend this mixture whenever the signal-to-noise ratio is expected to be low, for example, when carrying out northern analysis of low-abundance mRNAs or Southern hybridizations with single-copy sequences of mammalian DNA. However, in most other circumstances (Grunstein/Hogness hybridization (1975), Benton/Davis hybridization (1977), Southern hybridization (1975) of abundant DNA sequences, etc.), we recommend using 0.25% nonfat dried milk (0.05× BLOTTO; Johnson et al. 1984). This is much less expensive, easier to use than Denhardt's reagent, and, for these purposes, gives results that are equally satisfactory. In general, Denhardt's reagent is more effective for nylon membranes. The signal-to-noise ratio obtained with most brands of nylon membranes is higher with Denhardt's reagent than with BLOTTO. Nonfat dried milk is not recommended when using RNA probes or when carrying out northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase activity. For more information about blocking agents, see Table 9.1.

6. Blocking agents are usually included in both the prehybridization and hybridization solutions when nitrocellulose filters are used. However, when the nucleic acid is immobilized on nylon membranes, the blocking agents are often omitted from the hybridization solution, since high

TABLE 9.1 Blocking Agents Used to Suppress Background in Hybridization Experiments

Agent	Recommended uses
Denhardt's reagent	northern hybridizations hybridizations using RNA probes single-copy Southern hybridizations hybridizations involving DNA immobilized on nylon membranes

Denhardt's reagent (Denhardt 1966) is usually made up as a 50× stock solution, which is filtered and stored at −20°C. The stock solution is diluted tenfold into prehybridization buffer (usually 6× SSC or 6× SSPE containing 0.5% SDS and 100 μg/ml denatured, fragmented salmon sperm DNA). 50× Denhardt's reagent contains 5 g of Ficoll (Type 400, Pharmacia), 5 g of polyvinylpyrrolidone, 5 g of bovine serum albumin (Fraction V; Sigma), and H_2O to 500 ml.

BLOTTO	Grunstein/Hogness hybridization Benton/Davis hybridization all Southern hybridizations other than single-copy dot blots

1× BLOTTO (Bovine Lacto Transfer Technique Optimizer; Johnson et al. 1984) is 5% nonfat dried milk dissolved in water containing 0.02% sodium azide. It should be stored at 4°C and diluted 25-fold into prehybridization buffer before use. BLOTTO should not be used in combination with high concentrations of SDS, which will cause the milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent in northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase.

Caution: Sodium azide is poisonous. It should be handled with great care, wearing gloves, and solutions containing it should be clearly marked.

Heparin	Southern hybridization in situ hybridization

Heparin (Sigma H-7005 porcine grade II or equivalent) is dissolved at a concentration of 50 mg/ml in 4× SSPE or 4× SSC and stored at 4°C. It is used as a blocking agent at a concentration of 500 μg/ml in hybridization solutions containing dextran sulfate; in hybridization solutions that do not contain dextran sulfate, heparin is used at a concentration of 50 μg/ml (Singh and Jones, 1984).

Denatured, fragmented salmon sperm DNA	Southern and northern hybridizations

Salmon sperm DNA (Sigma type III sodium salt) is dissolved in water at a concentration of 10 mg/ml. If necessary, the solution is stirred on a magnetic stirrer for 2–4 hours at room temperature to help the DNA to dissolve. The concentration of NaCl is adjusted to 0.1 M, and the solution is extracted once with phenol and once with phenol:chloroform. The aqueous phase is recovered, and the DNA is sheared by passing it 12 times rapidly through a 17-gauge hypodermic needle. The DNA is precipitated by adding 2 volumes of ice-cold ethanol. It is then recovered by centrifugation and redissolved at a concentration of 10 mg/ml in water. The OD_{260} of the solution is determined and the exact concentration of the DNA is calculated. The solution is then boiled for 10 minutes and stored at −20°C in small aliquots. Just before use, the solution is heated for 5 minutes in a boiling-water bath and then chilled quickly in ice water. Denatured, fragmented salmon sperm DNA should be used at a concentration of 100 μg/ml in prehybridization solutions.

concentrations of protein are believed to interfere with the annealing of the probe to its target. This quenching of the hybridization signal is particularly noticeable when oligonucleotides or probes less than 100 nucleotides in length are used.

7. In the presence of 10% dextran sulfate or 10% polyethylene glycol, the rate of hybridization is accelerated approximately tenfold (Wahl et al. 1979; Renz and Kurz 1984; Amasino 1986) because nucleic acids are excluded from the volume of the solution occupied by the polymer and their effective concentration is therefore increased. Although dextran sulfate and polyethylene glycol are useful in circumstances where the rate of hybridization is the limiting factor in detecting rare sequences (e.g., northern or genomic Southern blots), they are of no benefit when screening bacterial colonies or bacterial plaques. In addition, they can sometimes lead to high backgrounds, and hybridization solutions containing them are always difficult to handle because of their viscosity. We therefore recommend that dextran sulfate and polyethylene glycol not be used unless the rate of hybridization is very slow, the filter contains very small amounts of DNA, or the amount of radiolabeled probe is limiting.

8. To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6× SSC or 6× SSPE) at a temperature that is 20–25°C below the melting temperature (T_m). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, 6× SSPE is preferred because of its greater buffering power.

9. In general, the washing conditions should be as stringent as possible (i.e., a combination of temperature and salt concentration should be chosen that is approximately 12–20°C below the calculated T_m of the hybrid under study). The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the probe of interest and then washed under conditions of different stringencies.

10. To minimize background problems, it is best to hybridize for the shortest possible time using the minimum amount of probe. For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^7 cpm/μg or greater) should be used and hybridization should be carried out for 12–16 hours at 68°C in aqueous solution or for 24 hours at 42°C in 50% formamide. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^8 cpm/μg or greater).

11. *Useful facts:*

    a. The T_m of the hybrid formed between the probe and its target may be estimated from the following equation (Bolton and McCarthy 1962):

$$T_m = 81.5°C + 16.6(\log_{10}[Na^+]) + 0.41(\text{fraction G + C}) - 0.63(\%\ \text{formamide}) - (600/l)$$

where l = the length of the hybrid in base pairs.
This equation is valid for:

- Concentrations of Na^+ in the range of 0.01 M to 0.4 M. It predicts T_m less accurately in solutions of higher $[Na^+]$.
- DNAs whose G + C content is in the range of 30% to 75%. Note that the depression of T_m in solutions containing formamide is greater for poly(dA:dT) (0.75°C/1% formamide) and less for DNAs rich in poly(dG:dC) (0.50°C/1% formamide) (Casey and Davidson 1977).

The equation applies to the "reversible" T_m that is defined by optical measurement of hyperchromicity at OD_{257}. The "irreversible" T_m, which is more important for autoradiographic detection of DNA hybrids, is usually 7–10°C higher than that predicted by the equation.
Similar equations have been derived for:

i. RNA probes hybridizing to immobilized RNA (Hodkin and Knudson 1985)

$$T_m = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58(\text{fraction G + C}) + 11.8(\text{fraction G + C})^2 - 0.35(\%\ \text{formamide}) - (820/l)$$

ii. DNA:RNA hybrids (Casey and Davidson 1977)

$$T_m = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58(\text{fraction G + C}) + 11.8(\text{fraction G + C})^2 - 0.50(\%\ \text{formamide}) - (820/l)$$

Comparison of these equations shows that the relative stability of nucleic acid hybrids decreases in the following order: RNA:RNA (most stable), RNA:DNA (less stable), and DNA:DNA (least stable). In aqueous solutions, the T_m of a DNA:DNA hybrid is approximately 10°C lower than that of the equivalent RNA:RNA hybrid. In 80% formamide, the T_m of an RNA:DNA hybrid is approximately 10°C higher than that of the equivalent DNA:DNA hybrid.

    b. The T_m of a double-stranded DNA decreases by 1–1.5°C with every 1% decrease in homology (Bonner et al. 1973).

The above equations apply only to hybrids greater than 100 nucleotides in length. The behavior of oligonucleotide probes is described in detail in Chapter 11.

For a general discussion of hybridization of nucleic acids bound to solid supports, see Meinkoth and Wahl (1984).

Hybridization of Radiolabeled Probes to Nucleic Acids Immobilized on Nitrocellulose Filters or Nylon Membranes

Although the method given below deals with RNA or DNA immobilized on nitrocellulose filters, only slight modifications are required to adapt the procedure to nylon membranes. These modifications are noted at the appropriate places in the text.

1. Prepare the prehybridization solution appropriate for the task at hand. Approximately 0.2 ml of prehybridization solution will be required for each square centimeter of nitrocellulose filter or nylon membrane.

   The prehybridization solution should be filtered through a 0.45-micron disposable cellulose acetate filter (Schleicher and Schuell Uniflow syringe filter No. 57240 or equivalent).

   Prehybridization solutions

   For detection of low-abundance sequences:

   Either

   6× SSC (or 6× SSPE)
   5× Denhardt's reagent
   0.5% SDS
   100 μg/ml denatured, fragmented salmon sperm DNA

   or

   6× SSC (or 6× SSPE)
   5× Denhardt's reagent
   0.5% SDS
   100 ng/ml denatured, fragmented salmon sperm DNA
   50% formamide

   For preparation of Denhardt's reagent and denatured, fragmented salmon sperm DNA, see Table 9.1.

   Formamide. Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman No. 1 paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

   For detection of moderate- or high-abundance sequences:

   Either

   6× SSC (or 6× SSPE)
   0.05× BLOTTO

   or

   6× SSC (or 6× SSPE)
   0.05× BLOTTO
   50% formamide

   For preparation of BLOTTO, see Table 9.1.

When ^{32}P-labeled cDNA or RNA is used as a probe, poly(A)$^+$ RNA at a concentration of 1 μg/ml may be included in the prehybridization and hybridization solutions to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA.

2. Float the nitrocellulose filter or nylon membrane containing the target DNA on the surface of a tray of 6× SSC (or 6× SSPE) until it becomes thoroughly wetted from beneath. Submerge the filter for 2 minutes.

3. Slip the wet filter into a heat-sealable bag (e.g., Sears Seal-A-Meal or equivalent). Add 0.2 ml of prehybridization solution for each square centimeter of nitrocellulose filter or nylon membrane.

   Squeeze as much air as possible from the bag. Seal the open end of the bag with the heat sealer. Incubate the bag for 1–2 hours submerged at the appropriate temperature (68°C for aqueous solvents; 42°C for solvents containing 50% formamide).

   Often, small bubbles of air form on the surface of the filter as the temperature of the prehybridization solution increases. It is important that these bubbles be removed by occasionally agitating the fluid in the bag; otherwise, the components of the prehybridization solution will not be able to coat the filter evenly. This problem can be minimized by heating the prehybridization solution to the appropriate temperature before adding it to the bag.

4. If the radiolabeled probe is double-stranded, denature it by heating for 5 minutes at 100°C. Single-stranded probe need not be denatured. Chill the probe rapidly in ice water.

   Alternatively, the probe may be denatured by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, transfer the probe to ice water and add 0.05 volume of 1 M Tris·Cl (pH 7.5) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.

   For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^8 cpm/μg or greater) should be used. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^8 cpm/μg or greater).

5. Working quickly, remove the bag containing the filter from the water bath. Open the bag by cutting off one corner with scissors. Add the denatured probe to the prehybridization solution, and then squeeze as much air as possible from the bag. Reseal the bag with the heat sealer so that as few bubbles as possible are trapped in the bag. To avoid radioactive contamination of the water bath, the resealed bag should be sealed inside a second, noncontaminated bag.

   When using nylon membranes, the prehybridization solution should be *completely* removed from the bag and immediately replaced with hybridization solution. The probe is then added and the bag is resealed.

Hybridization solution for nylon membranes
6× SSC (or 6× SSPE)
0.5% SDS
100 μg/ml denatured, fragmented salmon sperm DNA
50% formamide (if hybridization is to be carried out at 42°C)

6. Incubate the bag submerged in a water bath set at the appropriate temperature for the required period of hybridization.

7. Wearing gloves, remove the bag from the water bath and immediately cut off one corner. Pour out the hybridization solution into a container suitable for disposal, and then cut the bag along the length of three sides. Remove the filter and immediately submerge it in a tray containing several hundred milliliters of 2× SSC and 0.5% SDS at room temperature.

   Important: Do not allow the filter to dry out at any stage during the washing procedure.

8. After 5 minutes, transfer the filter to a fresh tray containing several hundred milliliters of 2× SSC and 0.1% SDS and incubate for 15 minutes at room temperature with occasional gentle agitation.

   If short oligonucleotides are used as probes, washing should be carried out only for brief periods (1–2 minutes) at the appropriate temperature. For a discussion of the stability of hybrids involving oligonucleotides, see Chapter 11.

9. Transfer the filter to a flat-bottom plastic box containing several hundred milliliters of fresh 0.1× SSC and 0.5% SDS. Incubate the filter for 30 minutes to 1 hour at 37°C with gentle agitation.

10. Replace the solution with fresh 0.1× SSC and 0.5% SDS, and transfer the box to a water bath set at 68°C for an equal period of time. Monitor the amount of radioactivity on the filter using a hand-held minimonitor. The parts of the filter that do not contain DNA should not emit a detectable signal. You should not expect to pick up a signal on the minimonitor from filters containing mammalian DNA that has been hybridized to single-copy probes.

11. Briefly wash the filter with 0.1× SSC at room temperature. Remove most of the liquid from the filter by placing it on a pad of paper towels.

12. Place the damp filter on a sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the filter. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with the radioactive ink.

    Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot (>2000 cpe on a hand-held minimonitor), hot (>500 cps on a hand-held minimonitor), and cool (>50 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

13. Cover the filter with a second sheet of Saran Wrap, and expose the filter to X-ray film (Kodak XAR-2 or equivalent) to obtain an autoradiographic image (see Appendix B). The exposure time should be determined empirically. However, single-copy sequences in mammalian genomic DNA can usually be detected after 16–24 hours of exposure at −70°C with an intensifying screen.

Hybridization of Radiolabeled Oligonucleotides to Genomic DNA

Oligonucleotide probes as short as 17 nucleotides in length may be used to detect single-copy sequences in restriction digests of eukaryotic genomic DNA that have been transferred to solid supports. As discussed in Chapter 11, hybrids of this length are stable enough to be detected in practice only if they are perfectly matched. Duplexes with a single base-pair mismatch are significantly less stable and dissociate at a lower temperature than their perfectly matched counterparts (Wallace et al. 1979; Ikuta et al. 1987). It has therefore been possible to use oligonucleotides of defined sequence to probe fetal DNA for the presence of specific point mutations that cause conditions such as sickle-cell anemia (Conner et al. 1983), certain thalassemias (Orkin et al. 1983; Pirastu et al. 1983), and α_1-antitrypsin deficiency (Kidd et al. 1983); to screen DNA extracted from tumor cells for mutations in oncogenes (Bos et al. 1984, 1985, 1987; Forrester et al. 1987; Rodenhuis et al. 1987); and to analyze highly polymorphic loci, for example, the major histocompatibility complex class I genes (Gelichter et al. 1986).

The methods used when hybridizing with oligonucleotide probes are similar to those described earlier in this chapter. However, attention should be paid to the following points:

1. Because of the small size of the target sequence, a minimum of 30 μg of mammalian genomic DNA should be applied to each lane of the agarose gel.
2. The sequences of oligonucleotides used as probes should be long enough to be unique within the target genome (17 nucleotides for the mammalian genome) and short enough to allow the detection of mismatches under the conditions of hybridization used. Typically, oligonucleotides used for screening mammalian genomic DNA are 19–21 nucleotides in length.
3. When used to detect point mutations, oligonucleotides are used in pairs; one member of the pair is perfectly homologous to the mutated gene sequence and the other is homologous to the wild-type sequence. Usually, the members of the pair differ in sequence by only one nucleotide. Before embarking on an analysis of genomic DNA with these probes, it is essential to establish hybridization and washing conditions using cloned fragments of DNA of known sequence that are homologous to each member of the pair of oligonucleotides. These methods are discussed in detail in Chapter 11. Reconstruction experiments, in which known amounts of the control DNAs are added to a large excess of genomic DNA (at least 30 μg), are then used to test the sensitivity of the system.
4. Oligonucleotides are radiolabeled by [γ-^{32}P]ATP and bacteriophage T4 polynucleotide kinase (see Chapter 11). These probes tend to hybridize nonspecifically to high-molecular-weight DNA immobilized on nitrocellulose filters or nylon membranes, producing a smear toward the top of the autoradiograph. It is therefore important to choose a restriction enzyme (or a combination of restriction enzymes) that yields a hybridizing fragment whose size is not greater than 5 kb.
5. After electrophoresis, the fragments of genomic DNA may be transferred to a solid support by the conventional Southern transfer technique or immobilized within the agarose gel itself by dehydration (Studencki and Wallace 1984). Although DNA immobilized within the gel appears to give somewhat stronger hybridization signals than DNA attached to a solid support, it cannot be hybridized sequentially to many different probes. This is a severe disadvantage when the amount of genomic DNA is limited (as is often the case in prenatal diagnosis, for example). We therefore recommend that the genomic DNA be transferred to a nylon membrane such as Nytran (Schleicher and Schuell) or GeneScreen (du Pont).
6. Wherever possible, negative and positive hybridization controls should be included in each gel.
7. Oligonucleotides may also be used to detect rare transcripts in northern blots that contain 30 μg of total cellular RNA (Zeff et al. 1986) or 6 μg of poly(A)$^+$ RNA (Gelichter et al. 1986).

Removal of Radiolabeled Probes from Nitrocellulose Filters and Nylon Membranes

Probes become irreversibly bound if nitrocellulose filters and nylon membranes are allowed to dry. Therefore, every effort should be made to ensure that the solid supports remain wet at all stages during hybridization, washing, and exposure to X-ray film.

REMOVING PROBES FROM NITROCELLULOSE FILTERS

1. Heat several hundred milliliters of 0.05× SSC, 0.01 M EDTA (pH 8.0) to boiling. Remove the fluid from the heat and add SDS to a final concentration of 0.1%. Immerse the filter in the hot elution buffer for 15 minutes.

2. Repeat step 1 with a fresh batch of boiling elution buffer.
   Important: Do not allow the filter to dry when transferring it between batches of hot elution buffer.

3. Rinse the filter briefly in 0.01× SSC at room temperature. Remove most of the liquid from the filter by placing it on a pad of paper towels.

4. Sandwich the damp filter between two sheets of Saran Wrap, and apply it to X-ray film to check that all of the probe has been removed.

5. The filter may now be dried, wrapped loosely in aluminum foil, and stored under vacuum at room temperature until needed.

REMOVING PROBES FROM NYLON MEMBRANES

1. Either

   - immerse the membrane in several hundred milliliters of 1 mM Tris·Cl (pH 8.0), 1 mM EDTA (pH 8.0), 0.1× Denhardt's reagent (see Table 9.1) for 2 hours at 75°C, or

   - immerse the membrane in 50% formamide, 2× SSPE for 1 hour at 65°C.

2. Rinse the membrane briefly with 0.1× SSPE at room temperature. Remove most of the liquid from the membrane by placing it on a pad of paper towels.

3. Sandwich the damp membrane between two sheets of Saran Wrap, and apply it to X-ray film to check that all of the probe has been removed.

4. The membrane may now be dried, wrapped loosely in aluminum foil, and stored under vacuum at room temperature until needed.

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